Detection of single *Dactylogyrus* spp. in DNA extracted from infected gill tissue of fishes using Polymerase Chain Reaction

Mozhdeganlou, Z.[1]; Ebrahimzadeh Mousavi, H.[1]*; Shayan, P.[2]; Soltani, M.[1]; Ebrahimzadeh, E.[2] and M. Rostami[1]

[1]Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. [2]Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Key Words:
Dactylogyrus spp.; gill; goldfish; PCR.

Correspondence
Ebrahimzadeh Mousavi, H.
Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, P. O. Box: 14155-6453, Tehran, Iran.
Tel: +98(21)61117000
Fax: +98(21)66933222
Email: hmosavi@ut.ac.ir

Received: 14 October 2010,
Accepted: 01 January 2011

Abstract

Dactylogyrus spp. are monogenean worms found mostly as ectoparasites on the gills of several fish species, including carp and goldfish. These parasites are commonly detected by microscopic analysis of the gill lamellae, but this is time-consuming and technically difficult. In contrast to this conventional method, molecular techniques provide specific, sensitive and safe detection of parasites. In the present study, polymerase chain reaction (PCR) and subsequent DNA sequencing were used to detect *Dactylogyrus* spp. Specific common primers were designed to amplify the ITS-1 region of the *rRNA* gene of *Dactylogyrus* spp. *Dactylogyrus* worms were collected from 100 goldfishes and identified using a dissection microscope. Then, single worms were used for DNA extraction. To evaluate the PCR, a single parasite was added to a parasite-free gill, which then had its DNA extracted. Subsequently, the PCR products were purified and sequenced. Comparison of the nucleotide sequences of the PCR products with GenBank sequences showed that there was 100% homology with sequences from two *Dactylogyrus* spp., namely *Dactylogyrus vastator* and *Dactylogyrus dulkeiti* (registered under accession numbers AJ 564159 and AJ 564126, respectively). The results obtained from sequence analyses were consistent with species identification by microscopy. Therefore, the results show that it is possible to develop a sensitive and precise PCR method for the detection of *Dactylogyrus*-infected fish using DNA extracted from the whole gill.

Introduction

Dactylogyrids are monogenean parasites that infect the gills of cyprinid fishes (Koskivaara *et al*., 1991; Ogawa, 1994; Simkova *et al*., 2007). The infection is highly host-specific (Whittington, 1998; Whittington *et al*., 2000; Xiao-Qin *et al*., 2000; Simkova *et al*., 2007). Infection causes thickening of the gill epithelium that impairs respiratory function, negatively affects growth and can even cause death, especially in small carps (Thoney and Hargis, 1991). Parasites with a direct life cycle, such as monogeneans, can reach epizootic levels very quickly when hosts and parasites are confined close together (Thoney *et al*., 1991; Blazek *et al*., 2008).

A large number of different fish species, particularly goldfishes, are imported every year into Iran from the Far East (e.g., China), Russia and Eastern Europe (Shamsi *et al*., 2009). Poor controls of importation permit the transport of parasites with direct life cycles between different countries, potentially leading to increases in the prevalence of infections (Ebrahimzadeh Mousavi *et al*., 2009). Therefore, health quality control of imported fishes is of paramount importance (Whittington and Chong, 2007). Many *Dactylogyrus* spp. have been reported in Iranian freshwater fish, and some of these have been introduced and transmitted via imported goldfish (Jalali and Monlar, 1990a, 1990b; Monlar and Jalali, 1992; Gussev *et al*., 1993; Ebrahimzadeh Mousavi *et al*., 2009; Shamsi *et al*., 2009). Present guidelines order the mandatory quarantine of imported fish for two weeks, but these measures are probably inadequate to prevent the spread of certain infections (Ebrahimzadeh Mousavi *et al*., 2009). Therefore, the import of ornamental fishes requires the highest levels of sanitation and health quality control. Health screening of imported fish needs to be precise and sensitive to detect *Dactylogyrus* spp. Microscopic analysis of gill lamellae is the traditional method used to detect and identify *Dactylogyrus* parasites. However, *Dactylogyrus* spp. are very small (usually less than 1 mm) and their collection from the gills requires optical instruments, and the identification of a single

Dactylogyrus worm can be difficult. The precise identification of these organisms is essential for understanding their epidemiology. Due to the lack of reliable information regarding the epidemiology of *Dactylogyrus* spp. in Iran, it is not possible to speculate on the sources of infection, and further investigations using molecular techniques and DNA sequence analyses should be useful for comprehending the molecular taxonomy and phylogeny of *Dactylogyrus* spp. (Shamsi *et al*., 2009).

In the present study, a polymerase chain reaction (PCR) method was developed to enable the identification of virtually all species of *Dactylogyrus*. The DNA was extracted from individual *Dactylogyrus* organisms isolated from the gills of goldfishes. This DNA was amplified using primers designed to sequences that flank the ITS-1 region of the *rRNA* gene, and subsequently sequenced. Moreover, using this PCR technique, the present study shows that it is capable of detecting a single worm that has been experimentally added to a gill.

Materials and Methods

Sampling and microscopic examination

A total of 100 goldfish (*Carassius auratus*) with a fork length of 4–5 cm and approximately 10 g weight were purchased from different pet shops in Tehran, and transferred alive in their original water to our laboratory (Aquatic Animal Health Department, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran). Wet mounts from both the left and right gill arches were examined for each fish. Firstly, the gills were washed with their original water to remove blood, and then analyzed for the presence of parasites using dissection and light microscopes. Each parasite was collected from the gills and the general morphology, such as sclerotized and copulatory organs, was photographed using a digital camera (Sony, SSC-DC80P No. 401182) under the light microscope. Identification of *Dactylogyrus* spp. using light microscopy was performed based on morphological (hook, marginal hook, bars and copulatory organ shape and number) characteristics of the parasite according to existing keys reported in Yamaguti (1961). Then, the isolated parasite was transferred into a sterile tube containing 20 µl of double distilled water for DNA extraction. To evaluate the capability of the newly developed PCR for detecting a parasite in the whole gill, a single parasite was added to parasite-free gill tissue. The DNA was then extracted from this gill tissue and used for PCR. A parasite-free gill taken from a healthy fish was used as negative control.

DNA extraction

DNA was extracted from individual parasites, parasite-free gill tissue (negative control) and gill tissue that had had a single parasite added, using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Briefly, the samples were lysed in 180µl lysis buffer, and then the proteins were degraded with 20µl proteinase K for 10 min at 55°C. To each sample was added 360µl binding buffer and these were incubated at 70°C for 10 min. Then 270µl ethanol (96%) was added to the solution before shaking, and transferring the whole volume to the MBST-column. The MBST-column was centrifuged, washed twice with 500 µl washing buffer, and then the DNA was eluted from the carrier using 80 µl elution buffer.

PCR amplification

The amplification of DNA was performed using two primers designed against sequences that flank the ITS-1 region of the *rRNA* gene of the worm. The sense primer was designed from the nucleotide sequence at position 498 to 515 of the *18S rRNA* gene (5`-CTG CGG AAG GAT CAT TAT C-3`), while the antisense primer was designed from the nucleotide sequence at position 1019 to 1028 of the *5.8S rRNA* gene (5`-GAT CCA CCA CTT GCA GTT GT-3`). These primers can amplify the corresponding sequences registered in GenBank for *Dactylogyrus zandati* (AJ 564165), *Dactylogyrus vastator* (AJ 564159), *Dactylogyrus wunderi* (AJ 566464), *Dactylogyrus vistulae* (AJ 564162, AJ 564161 and AJ 64160), *Dactylogyrus sphyrna* (AJ 54155), *Dactylogyrus similes* (AJ 564153), *Dactylogyrus rasissimus* (AJ 564151), *Dactylogyrus ramulosus* (AJ 564150), *Dactylogyrus propinquus* (AJ 564147), *Dactylogyrus lamellatus* (AJ 564139), *Dactylogyrus intermedius* (AJ 564139), *Dactylogyrus inexpectatus* (AJ 564138), *Dactylogyrus hemiamphibothrium* (AJ 564137), *Dactylogyrus finitimus* (AJ 564133), *Dactylogyrus fallax* (AJ 564132), *Dactylogyrus falcatus* (AJ 54130), *Dactylogyrus extensus* (AJ 564129), *Dactylogyrus dulkeiti* (AJ 564126), *Dactylogyrus crucifer* (AJ 564122), *Dactylogyrus chranilowi* (AJ 564117), *Dactylogyrus caballeroi* (AJ 564114), *Dactylogyrus borealis* (AJ 564113), *Dactylogyrus auriculatus* (AJ 564117), *Dactylogyrus amphibothrium* (AJ 564110), *Dactylogyrus alatus* (AJ 564109) and *Dactylogyrus achmerowi* (AJ 564108). The PCR was performed in a total volume of 100 µl, which included 10 µl of 10× PCR buffer (Cina Gene, Iran), 2.5 U Taq polymerase (Fermentas), 20 pg of each primer, 1.5 mM $MgCl_2$ and 15 µl DNA sample (100–200 ng). The amplification was carried out in a thermocycler (MWG, Germany) using the following conditions: 94°C for 5 min, followed by 38 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 45 s, then a final extension step of 72°C for 10 min. The PCR products were analyzed on a 1.5% agarose gel in 0.5× TBE buffer (for 1 l of aqueous 5× TBE buffer: 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA; pH 8.0) and visualized using ethidium bromide and a UV illuminator.

PCR product purification and sequencing

PCR products were purified from the salts and proteins using a PCR purification kit (MBST, Iran). Briefly, 200µl binding buffer was added to 100µl PCR product solution. After adding 150µl ethanol (96%) to the sample, the mixture was applied to the column. The column was washed twice with washing buffer, and PCR product was eluted from the column using 100µl elution buffer. Sequencing was performed from both sides of each PCR product by the Kawsar Biotech Company in Iran using a method based on Sanger (1977).

Results and Discussion

Dactylogyrus parasites were identified in 17 out of 100 fish samples. Microscopic analysis revealed that 17 fishes were infected with two species, namely *D. vastator* and *D. dulkeiti*.

Species identification was based only on morphological features, which is subjective, and means that proper preservation, staining and fixation of inner organs are required (Strona *et al*., 2009). Since *Dactylogyrus* spp. are very small (usually less than 1 mm) and the collection from the gill requires optical instruments and a high level of technical ability and experience, the identification of a single *Dactylogyrus* spp. worm in a fish can be problematic.

PCR has been used to obtain sequences for *Dactylogyrus* spp. and other monogenean parasites for phylogenetic analyses (Jovelin and Justine, 2001; Justine *et al*., 2002; Simkova *et al*., 2004; Wu *et al*., 2005; Plaisance *et al*., 2005; Simkova *et al*., 2007). In the present study, we showed that it is possible to extract the DNA from a single *Dactylogyrus* parasite and perform PCR on this sample. DNA was extracted from individual parasites, parasite-free gill tissue (negative control) and gill tissue that had had a single parasite added, and these samples were PCR-amplified separately. The results showed an expected PCR product of 532 nucleotides in length in the samples from the individual parasites and where single parasites had been added to parasite-free gills (Figure 1). The DNA extracted from the parasite added to a parasite-free gill tissue was used to evaluate the capacity of the newly developed PCR assay for identifying *Dactylogyrus* parasites in the whole gill tissue. The DNA extracted from parasite-free gill tissue was used as negative control and revealed no PCR product as expected (Figure 1). PCR products were purified and then sequenced. After comparing the nucleotide sequences of the PCR products with GenBank sequences, two *Dactylogyrus* species, *D. vastator* and *D. dulkeiti*, were identified. Results from the PCR experiments were consistent with the results achieved by microscopic analysis.

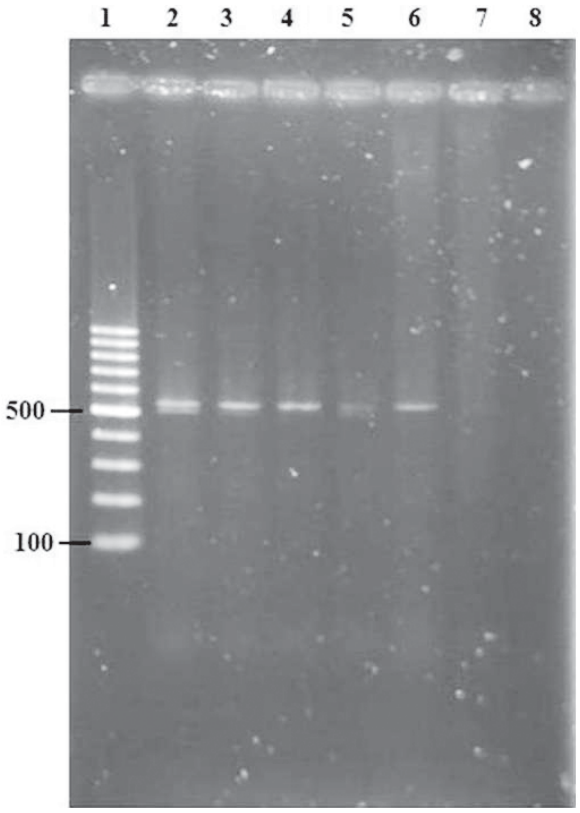

Figure 1: Agarose gel electrophoresis of PCR amplification products obtained from genomic DNA of *Dactylogyrus* spp. using *Dactylogyrus*-specific primers. Lane 1, 100 bp DNA marker; lane 2, *Dactylogyrus vastator* (single parasite); lane 3, single *D. vastator* parasite added to parasite-free gill tissue; lanes 4 and 5, *Dactylogyrus dulkeiti* (single parasite); lane 6, single *D. dulkeiti* parasite added to parasite-free gill tissue; lane 7, parasite-free gill tissue (negative control); lane 8, water (negative control).

More than 70 *Dactylogyrus* spp. have been reported in the freshwater fishes of Iran. Some of these have been transmitted into the country via the importation of goldfishes (Jalali and Monlar, 1990, 1990b; Monlar and Jalali, 1992; Gussev *et al*., 1993; Ebrahimzadeh Mousavi *et al*., 2009; Shamsi *et al*., 2009). We speculate that these non-native parasites are being introduced into Iran due to the limited health quality controls imposed on the importation of fishes (Ebrahimzadeh Mousavi *et al*., 2009). Despite the fact that these parasites display high host-specificity, the risk of introducing pathogenic *Dactylogyrus* spp. that can switch host to native fishes seems to be high. *Dactylogyrus* parasites have a direct life cycle that allows them to increase quickly in numbers in aquarium fish (Ebrahimzadeh Mousavi *et al*., 2009). Therefore, imported fish should be examined for *Dactylogyrus* spp. before exiting quarantine to prevent the spread of these parasites.

Since microscopic analysis of gill tissue is difficult and subjective, the newly developed PCR technique can replace this method. PCR is a simple, sensitive and

precise procedure. Therefore, we recommend that the Veterinary Organizations responsible for the sanitation and health quality controls of fishes improve their fish health guidelines by adopting molecular methods such as PCR for diagnosing fish parasites. To achieve this aim, the gills can be removed from randomly selected fish, the DNA can be extracted and analyzed by PCR using the primers described herein. This is the first study to detect *Dactylogyrus* spp. parasites using molecular identification techniques in Iran.

Acknowledgements

This study was supported financially by University of Tehran. The authors wish to thank Mrs. Narges Amini for her technical help.

References

1. Blazek, R.; Jarkovsky. J.; Koubkova, B. and Gelnar, M. (2008) Seasonal variation in parasite occurrence and microhabitat distribution of monogenean parasites of gudgeon Gobio gobio (L.). Helminthologia 45: 185-191.
2. Ebrahimzadeh Mousavi, H.A. (2003) Parasites of ornamental fish in Iran. Bul. Eur. Assoc. Fish. Pathol. 8: 297-300.
3. Ebrahimzadeh Mousavi, H.A.; Mehdizadeh, S.; Shoeibi, B.; Mokhayer, B.; Soltani, M.; Ahmadi, M. and Mirzargar, S. (2009) Gill ectoparasites of goldfish imported into Iran. Bull. Eur. Assoc. Fish. Pathol. 29: 69-74.
4. Gussev, A.V.; Jalali, B. and Molnar, K. (1993) Six new species of the genus Dactylogyrus (Monogenea: Dactylogyridae) from Iranian freshwater fishes. Zoosyst. Rossica. 2: 29-35.
5. Jalali, B.; Molnar, K. (1990a) Occurrence of monogeneans on freshwater fishes of Iran: Dactylogyridae from fish of natural waters and description of Dogelius mokhayeri sp. Parasit. Hung. 23: 27-32.
6. Jalal Band Molnar, K. (1990b) Occurrence of monogeneans on freshwater fishes in Iran: Dactylogyrus spp. on cultured fish. Acta Vet. Hung. 38: 239-242.
7. Jovelin, R.; Justine, J.L. (2001) Phylogenetic relationships within the polyopisthocotylean monogeneans (Platyhelminthes) inferred from partial 28S rDNA sequences. Int. J. Parasitol. 31: 393-401.
8. Justine, J.L.; Jovelin, R.; Neifar, L.; Mollaret, I.; Lim, L.H.S.; Hendrix, S.S. and Euzet, L. (2002) Pylogenetic position of the Bothitrematidae and Neocalceostomatidae (Monopisthocotylean Monogeneans) inferred from 28S rDNA sequences. Com. Parasitol. 69: 20-25.
9. Koskivaara, M.; Valtonen, E.T. and Prost, M. (1991) Dactylogyrids on the gills of roach in central Finland: features of infection and species composition. Int. J. Parasitol. 21: 565-572.
10. Molnar, K.; Jalali, B. (1992) Further monogeneans from Iranian freshwater fishes. Acta Vet. Hung. 40: 55-61.
11. Ogawa, K. (1994) Monogenean parasites of freshwater fishes of Hokkaido, Japan. Sci. Rep. Hokkaido. Fish Hatchery. 48: 59-67.
12. Plaisance, L.; Littlewood, D.T.J.; Olson, P.D. and Morand, S. (2005) Molecular phylogeny of gill monogeneans (Platyhelminthes, Monogenea, Dactylogyridae) and colonization of Indo-West Pacific butterflyfish hosts (Perciformes, Chaetodontidae). Zool. Scr. 34: 425-436.
13. Shamsi, S.; Jalali, B. and Aghazadeh Meshgi, M. (2009) Infection with Dactylogyrus spp. among introduced cyprinid fishes and their geographical distribution in Iran. I JV R. 10: 70-74.
14. Simkova, A.; Morand, S.; Jobet, E.; Gelnare, M. and Verneau, O. (2004) Molecular phylogeny of congeneric monogenean parasites (Dactylogyrus): a case of intrahost speciation. Evolution. 58: 1001-1018.
15. Simkova, A.; Pecinkova, M.; Rehulkova, E. and Vyskocilova, M. (2007) Dactylogyrus species parasitizing European Barbus species: morphometric and molecular variability. Parasitology. 134: 1-15.
16. Strona, G.; Stefani, F. and Galli, P. (2009) Field preservation of monogenean parasites for molecular and morphological analyses. Parasitol. Int. 58: 51-54.
17. Thoney, D.A.; Hargis, W.J. (1991) Monogenea (platyhelminthes) as hazards for fish in confinement. Ann. Rev. Fish Dis. 1: 133-153.
18. Whittington, I.D. (1998) Diversity “down under”: monogeneans in the Antipodes (Australia) with a prediction of monogenean biodiversity worldwide. Int. J. Parasitol. 28: 1481-1493.
19. Whittington, I.D.; Cribb, B.W.; Hamwood, T.E. and Halliday, J.A. (2000) Host-specificity of monogenean (platyhelminth) parasites: a role for anterior adhesive areas? Int. J. Parasitol. 30: 305-320.
20. Whittington, R.J.; Chong, R. (2007) Global trade in ornamental fish from Australian perspective: The case for revised import risk analysis and management strategies. Prev. Vet. Med. 81: 92-116.
21. Wu, X.Y.; Li, A.X.; Zhu, X.Q. and Xie, M.Q. (2005) Description of Pseudorhabdosynochus seabassi sp. n. (Monogenea: Diplectanidae) from Lates calcarifer and revision of the phylogenetic position of *Diplectanum grouperi* (Monogenea: Diplectanidae) based on rDNA sequence data. Folia. Parasitol. 52: 231-240.
22. Xiao-Qin, X.; Wei-Jun, W. and Cheng-Ping, L. (2000) The distribution of monogenean parasites on aquatic vertebrates inhabiting Chinese inland waters. Syst. Parasitol. 46: 151–155.
23. Yamaguti, S. (1961) Systema helminthum, Monogenea and Aspidocotylea. Vol. 4, Interscience publishers, New York, USA.